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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project seeks to identify and validate novel therapeutic targets for triple-negative breast cancer (TNBC). 681 genes showed consistent and highly significant overexpression in TNBC compared to receptor-positive cancers in 2 data sets. For two genes, 3 of the 4 siRNAs showed preferential growth inhibition in TNBC cells. These two genes were the low density lipoprotein receptor-related protein 8 (LRP8) and very low-density lipoprotein receptor (VLDLR). Exposure to their cognate ligands, reelin and apolipoprotein E isoform 4 (ApoE4), stimulated the growth of TNBC cells in vitro. Suppression of the expression of either LRP8 or VLDLR or exposure to RAP (an inhibitor of ligand binding) abolished ligand-induced proliferation. High-throughput protein and metabolic arrays revealed that ApoE4 stimulation rescued TNBC cells from serum-starvation induced up-regulation of genes involved in lipid biosynthesis, increased protein expression of oncogenes involved in the MAPK/ERK and DNA repair pathways, and reduced the serum-starvation induction of biochemicals involved in oxidative stress response and glycolytic metabolism. shLRP8 MDA-MB-231 xenografts had reduced tumor volume and increased levels of necrotic cells, in comparison to parental and shCON xenografts. These results indicate that LRP8-APOE signaling confers survival advantages to TNBC tumors under reduced nutrient conditions and during environmental stress. As such, they may serve as potential targets for the treatment of triple-negative breast disease.

15. SUBJECT TERMS
Therapeutic targets, triple-negative breast cancer, cancer metabolism.

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Table of Contents

INTRODUCTION

Triple-negative breast cancers lack the expression of estrogen and progesterone receptors and human epidermal growth factor receptors. As such, the treatment for these cancers is restricted to cytotoxic chemotherapy, which kills both normal and cancerous cells. The purpose and scope of this project is to identify and validate new therapeutic targets for the specific and tailored treatment of triple-negative breast cancer.

BODY

The results report on cumulative tasks completed during the entire research period. The results from tasks 1-4 were reported in the 2011 Annual Summary. The results from tasks 5-8 (partial completion of task 9) were reported in the 2012 Annual Summary. All results are summarized below with references to data that were included in previous annual summaries. **This report contains new data for the period from January 1, 2013 to May 31, 2013, the date of project completion and subsequent termination.**

Specific Aim	Task	Month(s)	Status
1	1	1	Completed
1	2	1-2	Completed
2	3	3-12	Completed
2	4	13-15	Completed
2	5	16-18	Completed
3	6	15-18	Completed
3	7	19	Completed
3	8	20-21	Completed
3	9	22-24	Completed

Specific Aim 1: To identify genes that are differentially overexpressed and amplified in triple-negative breast cancer (TNBC) vs. receptor-positive breast cancer (ER+/HER2- or ER-/HER2+).

Task 1: Unsupervised hierarchical gene clustering and class comparison analyses on 2 public gene expression datasets of human breast tumors, profiled on Affymetrix HG-U133A microarray.
Task 2: Analysis of comparative genomic hybridization (CGH) array data through circular binary segmentation method on 2 public datasets of human breast tumors.
Results: 684 overexpressed genes were found to be enriched in TNBC. Seventy-three TNBC genes (11%) were mapped to DNA segments that showed at least low level copy number gain in >10% of TNBC cases. These data suggest that DNA copy number gains may account for the mRNA overexpression of at least some of the genes that are overexpressed in TNBC (Table 1, 2011 Annual Summary).

Specific Aim 2: To determine the role of critical growth genes in the aggressive phenotype of triple-negative breast cancer cell lines.

Task 3: We will inhibit candidate genes in a panel of 19 breast cancer cell lines using a custom, high-throughput siRNA screen. We will observe the effect on cellular viability after

transfection with small-interfering RNA (siRNA), which down-regulates each candidate gene.

Task 4: The validation of target gene knockdown will be performed individually for the top 3 druggable genes in 3 TNBC cell lines (MDA-MB-231, MDA-MB-435, and MDA-MB-468).

Task 5: The effect of target gene knockdown on the function of TNBC cell lines will be assessed for the top druggable genes.

Results: siRNA screen optimization resulted in the determination of optimal large-scale screening conditions for the 18 breast cancer cell line panel (Table 2 and Table 3 from 2011 Annual Summary). As a result of successful siRNA screening, 684 candidates were whittled down to 27 gene hits which resulted in significant growth inhibition in triple-negative cell lines with $\geq$ 2 siRNAs/gene (Figure 1 and Table 4 from 2011 Annual Summary). Validation of target RNA and protein knockdown was performed with quantitative RT-PCR and Western blotting and siRNA-resistant plasmid rescue experiments (Figure 2 and Figure 3 from 2011 Annual Summary). As a result, 2 final top hits were identified, LRP8 and VLDLR. Further characterization of the ligands of these 2 transmembrane receptors revealed that isoform-dependent growth stimulation was observed with ApoE isoform 4. Exposure to reelin and ApoE isoform 4, ligands to LRP8 and VLDLR, stimulates the growth of ER-negative cells in vitro. The rate of cellular division was significantly increased, as evidenced in bromodeoxyuridine (BrdU) incorporation assays (Figure 4, 5, and 6 in 2011 Annual Summary, Figure 1 in 2012 Annual Summary). siLRP8 treatment abrogated this response, indicating that the stimulatory effect was centered around LRP8 – ApoE4 signaling. The rate of apoptosis was not significantly reduced with ApoE4 or siLRP8 treatment, indicating that proliferation was the functional endpoint for LRP8- and VLDLR-expressing TNBC cells which are stimulated by ApoE4. We also examined to effect of a mouse monoclonal antibody against LRP8 reduced the binding affinity of recombinant human LRP8 and ApoE4 using an ELISA binding assay (Figure 2 in 2012 Annual Summary).). In comparison, a mouse polyclonal antibody against the intracellular C-terminal region of LRP8 and mouse IgG antibody did not affect the binding of recombinant LRP8 and ApoE4 (Figure 2 in 2012 Annual Summary).

Further global analysis revealed that LRP8 – ApoE4 signaling deactivated lipid stereogenesis in response to nutrient starvation, suggesting that this signaling axis promotes cancer cell growth by momentarily reverting to catabolic pathways. This further indicates that LRP8 – ApoE4 allows TNBC cells to grow and survive using alternate metabolic pathways (Figure 1, 2, 3), such as glycolysis and the pentose phosphate pathway to meet energy demands (Figure 3 and 4 in 2012 Annual Summary).

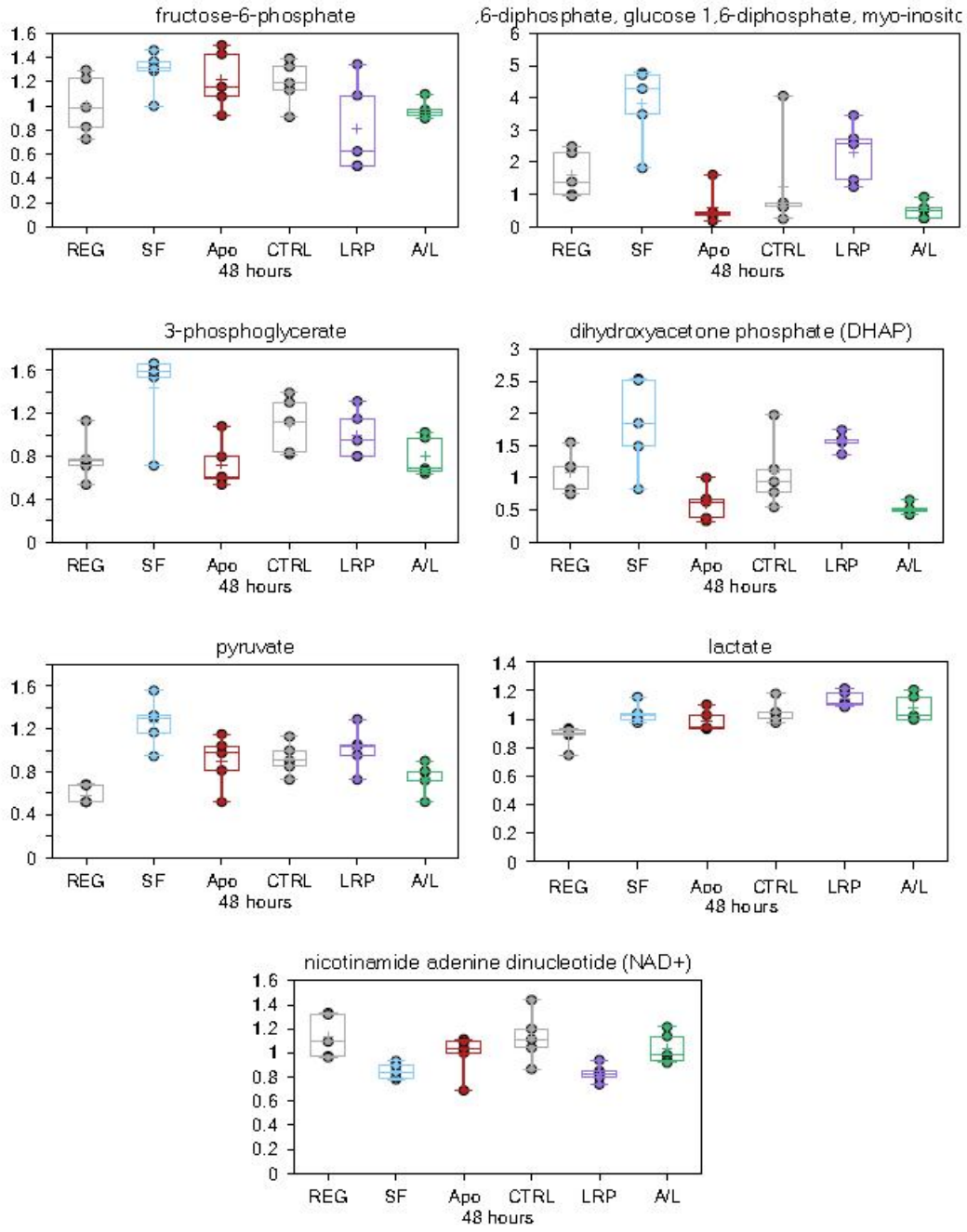

Figure 1. Glycolytic metabolism in BT-549 cells under serum-starved. The increase in intermediates of glycolysis suggest that TNBC cells utilize Warburg metabolism to grow despite nutrient conditions that are depleted of sustenance. ApoE4 stimulation resulted in the rescue of the Warburg effect and reduced the levels of glycolytic intermediates.

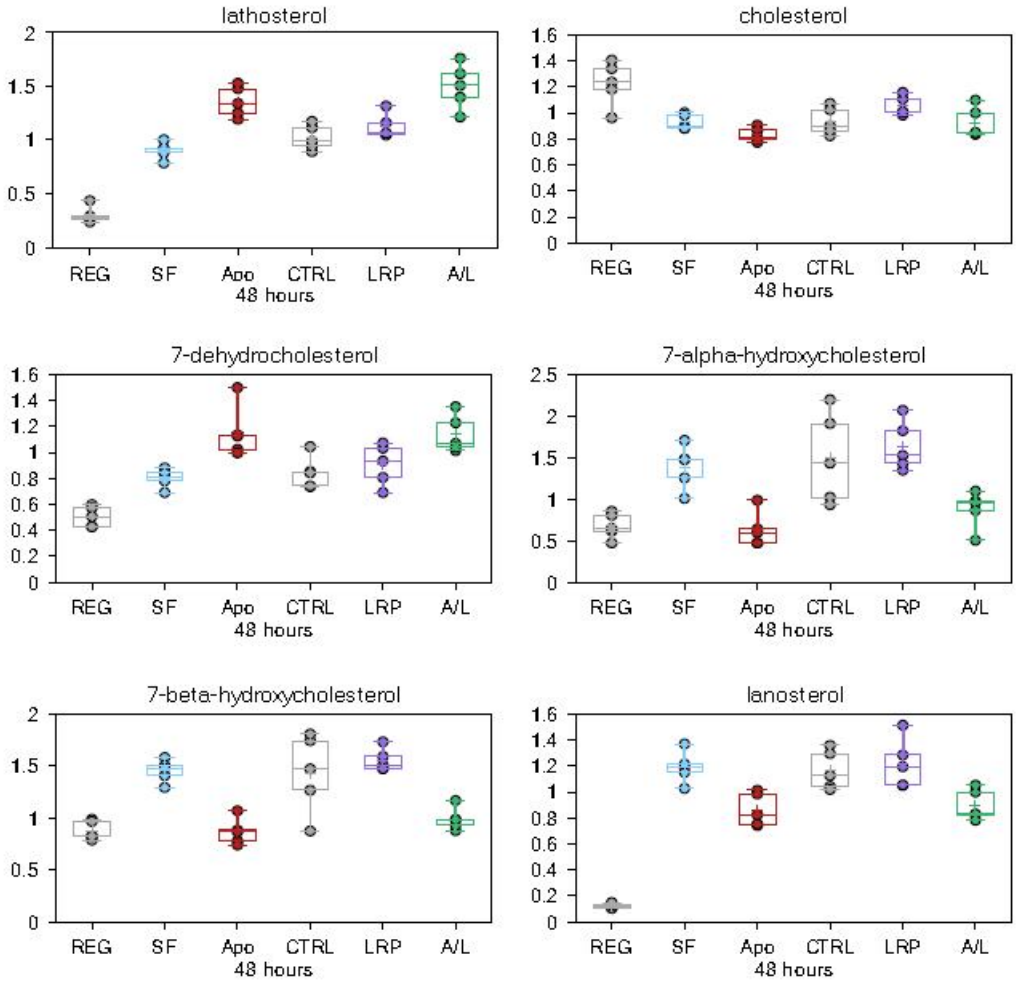

Figure 2. ApoE4-induced rescue of serum starvation resulting in reduction of cholesterol and fatty acid metabolites. As noted in gene expression profiling and confirmed in metabolic profiling, serum starvation affected levels of several cholesterol metabolites. ApoE4 rescued effects of serum starvation on likely oxidative stress metabolites 7α-hydroxycholesterol and 7β-hydroxycholesterol and the cholesterol precursor lanosterol. ApoE4 effects on cholesterol metabolism are related to the role of ApoE4 in regulation of cholesterol transport and metabolism.

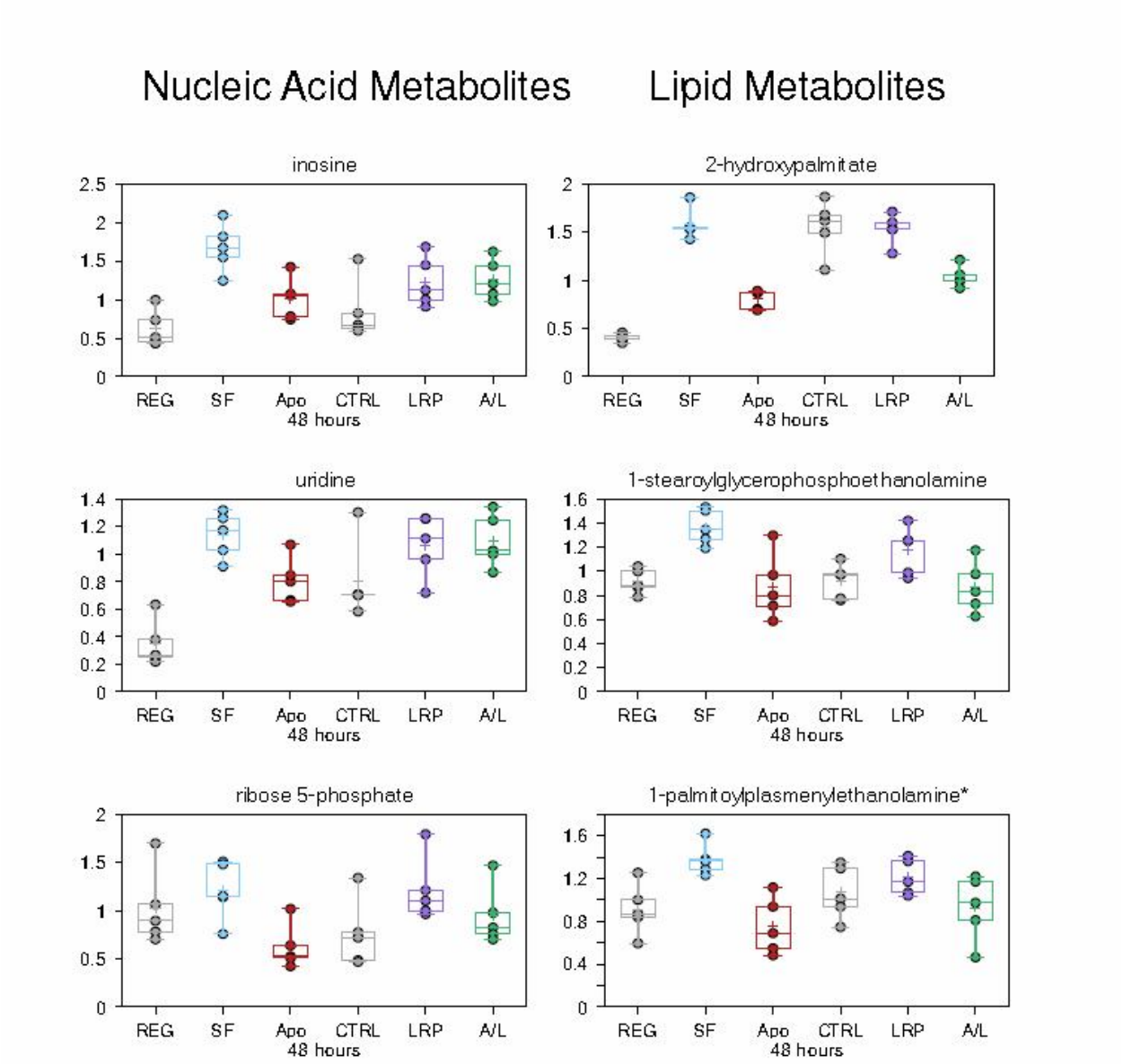

Figure 3. LRP8 knockdown reverses ApoE4-induced growth and restores pentose phosphate pathway. Nucleic acid metabolites, inosine, guanosine and uridine were more abundant in cells with serum-free media relative to regular media, and LRP knockdown further increased the levels of these nucleosides, while ApoE4 addition had the opposite effect. Intermediates to nucleotide synthesis that are components of the pentose phosphate pathway (ribose-5-phosphate and the ribulose-5-phosphate isobar) also had similar patterns. This pattern suggests that LRP8 may block ApoE rescue by impinging upon the pentose phosphate pathway and reverting the cellular response to serum-free metabolic responses.

Specific Aim 3: To determine whether silencing critical growth genes effects the growth of triple-negative breast cancer xenografts.

Task 6: Obtain Institutional Animal Care and Use Committee Protocol Approval (Office of Research Administration), Category 2 Protocol (little or no pain or distress protocol), import from The Jackson Laboratory (Stock: 002019, Strain: NU/J, Genotype: *A/A Tyrc/Tyrc Foxn1nu/Foxn1nu*), total 35 mice/experiment, 10 mice/group, 3 groups (shTARGET, shCONTROL, uninjected), total 3 experiments, 6 months/experiment.

Task 7: Establish orthotopic xenografts using MDA-MB-231 TNBC cells.

Task 8: In vivo gene silencing of target gene with shRNA delivery into the TNBC xenografts.

Task 9: Examination of orthotopic xenograft specimens and major organs.

Results: Results indicate that shLRP8 xenografts have reduced tumor volume, in comparison to parental or shCON MDA-MB-231 xenografts (Figure 4). The level of tumor necrosis is also elevated in shLRP8 xenografts. Interestingly, the number of lymphovascular invasions are increased in shLRP8 xenografts, suggests that loss of ApoE-mediated anti-angiogenic signaling through LRP8 increases the invasive potential of triple-negative tumor cells that are able to survive.

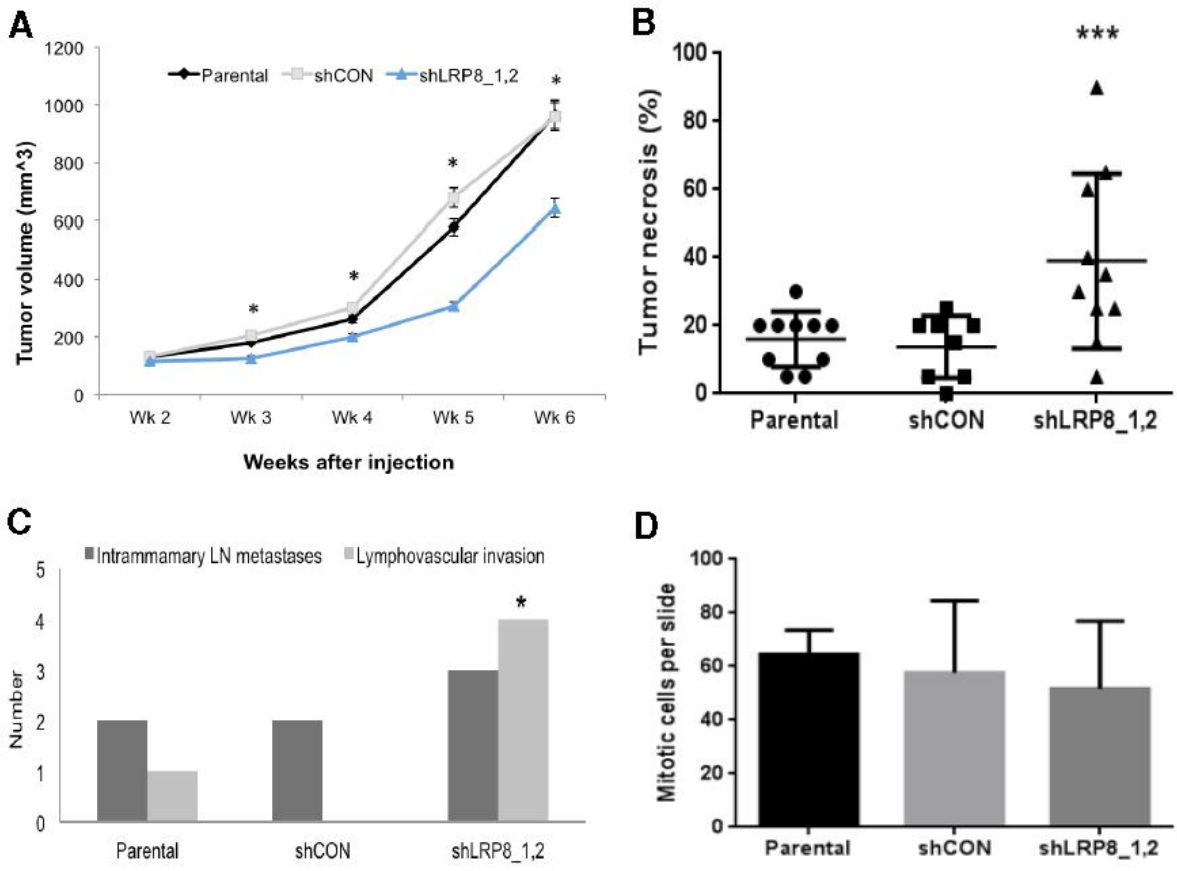

Figure 4. shLRP8 MDA-MB-231 xenograft study. (A) MDA-MB-231 parental, shCON, and shLRP8 xenografts were established in 4 week old nude mice (n=12 per group). (B) Tumor necrosis as a percentage of total tumor section per slide is shown. (C) Tumor sections were examined for the presence of intramammary lymph node metastases and sectioned nodes were examined for lymphovascular invasion. Raw number count is depicted in the first graph. (D) Mitotic cells were counted and the mean counts were tested. Mean tumor quantities of the three groups were tested with one-way ANOVA analysis. In all panels, * indicates $p<0.05$ and *** indicates $p<0.01$.

KEY RESEARCH ACCOMPLISHMENTS

- Validation of the LRP8 – ApoE4 signaling axis in the proliferation of TNBC cell lines
- Identification of altered fatty acid and glycolytic pathways in response to LRP8 – ApoE4 signaling in TNBC cell lines
- shLRP8 xenograft results indicate that *in vivo* depletion of LRP8 reduces tumor volume

REPORTABLE OUTCOMES

Awards

1. Genomics, Proteomics, and Imaging Poster session 2nd Prize: Identification of Novel Therapeutic Targets for Triple-Negative Breast Cancer, The University of Texas M. D. Anderson Cancer Center, 2010

2. M.D. Anderson Alumni and Faculty Association Graduate Student Award in Clinical/Translational Research, 2011

Abstracts

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Manuscript draft in progress

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CONCLUSION

The summarized work reveals potential new therapeutic targets for a subset of breast cancers which have limited therapeutic options. LRP8 and VLDLR are two surface membrane receptors that act as growth signals for triple-negative breast cancer may be targeted with targeted therapeutics. In response to LRP8 – ApoE4 signaling, downstream pathways are activated that result in altered metabolic adaptations which allow TNBC cells to grow in the absence of nutrients. *In vitro* and *in vivo* studies suggest that this signaling axis may serve as a targetable pair for triple-negative disease. Future directions include developing an epitope-specific antibody that disrupts the ligand-receptor pair with a chemical library screening process

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APPENDICES

- None